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Thrombospondin-1 promotes matrix homeostasis by interacting with collagen and lysyl oxidase precursors and collagen cross-linking sites**

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ABSTRACT

Fibrillar collagens of the extracellular matrix are critical for tissue structure and physiology, yet excessive or abnormal deposition of collagens is a defining feature of fibrosis. Regulatory mechanisms that act on collagen fibril assembly potentially offer new targets for anti-fibrotic treatments. Tissue weakening or altered collagen fibril morphologies, or both, are shared phenotypes of mice lacking matricellular thrombospondins. Thrombospondin-1 (TSP1) plays an indirect role in collagen homeostasis through interactions with matrix metalloproteinases and transforming growth factor-β1 (TGFβ1). We found that TSP1 also affects collagen fibril formation directly. Compared to skin from wild-type mice, skin from *Thbs1*$^{-/-}$ mice had reduced collagen crosslinking and reduced prolysyl oxidase (proLOX) abundance with increased conversion to catalytically active LOX. In vitro, TSP1 bound to both the C-propeptide domain of collagen I and the highly-conserved KGHR sequences of the collagen

triple-helical domain that participate in cross-linking. TSP1 also bound to proLOX and inhibited proLOX processing by bone morphogenetic protein-1 (BMP-1). In human dermal fibroblasts (HDFs), TSP1 and collagen I colocalized in intracellular vesicles and on extracellular collagen fibrils, whereas TSP1 and proLOX colocalized only in intracellular vesicles. Inhibition of LOX-mediated collagen crosslinking did not prevent the extracellular association between collagen and TSP1; however, treatment of HDFs with KGHR-containing, TSP1-binding, triple-helical peptides disrupted the collagen-TSP1 association, perturbed the collagen extracellular matrix, and increased myofibroblastic differentiation in a manner that depended on TGFβ receptor 1. Thus, the extracellular KGHR-dependent interaction of TSP1 with fibrillar collagens contributes to fibroblast homeostasis.

INTRODUCTION

Fibrillar collagens are abundant and essential components of the extracellular matrix (ECM) of connective tissues. The processing and secretion of collagen molecules and their initial assembly into fibrils has been studied extensively, yet knowledge of the cell and molecular mechanisms that regulate the organization of collagen fibrils within the ECM remains limited. This is of major importance for human health because fibrosis, the excessive and disorderly deposition of collagenous ECM by myofibroblasts as a result of tissue injury and repair, is a central and currently untreatable pathology in many chronic human diseases (*1*). Although the collagen family is quite extensive, the fibrillar collagens I, II, and III are the most abundant. Collagens II and III are homotrimers, with three identical α-chains and separate gene products for each type. Each assembles as a right-handed triple helix. Collagen I, the most widespread, differs in that it is comprised of two α1 chains and a single α2 chain. Collagen II is the principal collagen of cartilage, whereas collagen I predominates in bone and tendon (*2*). Collagens I and III both occur in skin and blood vessel walls, but collagen I is most abundant, and is the main focus of the present work.

The synthesis of fibrillar collagens involves multiple steps of processing and post-translational assembly (*2*). Each α-chain is translated as a preproprotein from which the secretory signal peptide is cleaved upon co-translational entry into the lumen of the endoplasmic reticulum (ER). This is followed by steps important for stable assembly of the triple-helix: post-translational hydroxylation of lysine and proline residues and glycosylation of lysines (*3*). The C-propeptide domain guides α-chain assembly into procollagen molecules, forming an in-register triple-helix and preventing premature intracellular fibril nucleation by restricting lateral packing (*4*). Upon secretion, the C-propeptide domain is

cleaved off mainly by bone morphogenetic protein 1 (BMP-1), and the N-propeptide is cleaved off by A Disintegrin and Metalloproteinase with Thrombospondin motifs 2 (ADAMTS2), generating mature collagen molecules, consisting of the N- and C-telopeptides and the central triple-helical domain, that are competent for fibril assembly. In parallel, secreted prolysyl oxidase (proLOX) is also cleaved by BMP-1 to yield active mature (mLOX), which catalyzes oxidative deamination of lysine residues in the collagen N- and C-telopeptides (*3, 5*). The resulting reactive lysine aldehydes target lysines or hydroxylysines of KGHR motifs within the triple-helix to cross-link and stabilize the assembled fibrils (*6*).

In common with collagens, thrombospondins (TSPs) are amongst the most ancient and conserved components of the ECM (*7*). TSPs are matricellular proteins that have context- and tissue-specific roles through interactions with cell-surface receptors, growth factors, and other ECM proteins (*8, 9*). There are five TSPs in mammals (TSP1–5; TSP5 is also known as COMP, cartilage oligomeric matrix protein) and, in both humans and mice, tissue abundance of TSP1 and TSP2 increases with ageing, during which the ECM becomes less elastic and more rigid (*10,11*). Rare polymorphisms in TSP1 have also been implicated in increased risk of cardiovascular disease (*12, 13*). TSP1 acts as an inhibitor of tumor growth (*14*) because it inhibits angiogenesis; however, the presence of TSP1 in tumor stroma may promote cell migration or ECM turnover, or both, leading to increased tumor metastasis (*15*). Increased abundance or extracellular deposition of TSPs, or both, also correlate with fibrotic events (*16-18*). Knockout mice have been reported for each individual TSP and show distinct tissue phenotypes and altered pathological susceptibilities that relate to altered cell-ECM interactions and signaling as well as effects on ECM properties (*19-23*). Mice lacking *Thbs1*, the gene that encodes TSP1, have increased vascular density in many tissues (*24, 25*), delayed wound healing, altered inflammatory responses, reduced abundance of active transforming growth factor-β (TGF-β) under various challenges, and increased susceptibility to tumor progression due to loss of the normal anti-angiogenic and p53-regulatory activities of TSP1 (*26-28*). An emerging coincident phenotype reported for *Thbs1*$^{-/-}$, *Thbs2*$^{-/-}$, *Thbs4*$^{-/-}$ and *Thbs5*$^{-/-}$ mice is disorganization of collagen fibril packing and altered fibril organization, manifested as increased cross-sectional areas of collagen fibrils in various tissues of young, healthy mice and correlated in several instances with mechanical weakening of the tissue (*21-23, 29, 30*). These phenotypes are reminiscent of tissue-specific alterations to collagen fibrils observed in dermis or tendons upon knockout of small leucine-rich proteoglycans such as decorin or biglycan (*31, 32*). Similarly, mice lacking LOX also show impaired collagen fibril organization (*33*). LOX is a vital, copper-dependent enzyme with roles in ECM integrity, vessel wall mechanotransduction and tumor progression, that initiates extracellular cross-linking of collagen or elastin (*34*).

Investigations of the molecular basis for the TSP-null phenotypes have emphasized indirect regulatory mechanisms. For example, the activities of matrix metalloprotease-2 (MMP-2) and MMP-9 are altered in *Thbs1*$^{-/-}$ and *Thbs2*$^{-/-}$ mice, and TSP1 and TSP2 bind to and inhibit the activities of these proteases in vitro (*35-37*). Cell type–specific effects on collagen transcription have also been noted: *Thbs1*$^{-/-}$ vascular cells have increased expression and secretion of collagen α1(I) and α2(I) chains (*38*), whereas the N-terminal domain of TSP1 increased collagen production and ECM assembly in vitro and in a sponge implant in vivo (*39*). Because TSP1 binds to and activates latent TGF-β, reduced TGF-β signaling in tissues of *Thbs1*$^{-/-}$ mice also affects ECM properties (*28, 40*). There remains a major gap in knowledge on the mechanisms of direct interaction of TSPs with fibrillar collagens and the importance of these in cell-ECM physiology. In vitro biochemical and electron microscopy studies have shown that several TSPs bind fibrillar collagens I, II, III and V, and TSP5 has also been shown to bind to collagen IX (*41, 42*). However, many of these studies relied on supra-physiological Zn^{2+} concentrations to demonstrate the protein-protein interactions (*43*).

In view of the potential physiological importance of TSP-collagen interactions in ECM reorganization during ageing, tumour progression, tissue fibrosis, and other disease states, we have focused on TSP1 and set out to elucidate the molecular basis of interactions between TSP1 and fibrillar collagen. We quantified the status of fibrillar collagens and LOX in the skin of *Thbs1*$^{-/-}$ mice and examined the binding of TSP1 to native and denatured collagens, recombinant collagens and collagen-derived peptides, and a library of synthetic triple-helical peptides designated the “Collagen Toolkit” (*44*). The Toolkit peptides are homotrimeric, which simplifies their synthesis. However, because there is much sequence homology between collagens I, II, and III, observations made using Toolkits II and III can often be applied directly to collagen I. We applied these findings to the physiological context of human dermal fibroblasts to examine the functional and regulatory roles of these interactions. Our data reveal that TSP1 participates in both intracellular and extracellular associations with fibrillar collagens that impact collagen cross-linking and fibroblast phenotype.

RESULTS

Abundance of collagen I and LOX is altered in the skin of *Thbs1*$^{-/-}$ mice

Collagen fibrils in the dermis of young, healthy *Thbs1* knockout (*Thbs1*$^{-/-}$) mice are characterized by irregular packing and increased cross-sectional areas (*29*). To investigate the underlying molecular basis, we examined eight-week old, male C57BL/6 and C57BL/6*Thbs1*$^{-/-}$ mice (*38*). We confirmed the *Thbs1*$^{-/-}$ status of the latter mice by immunoblotting extracts from spleen, a tissue that normally has high abundance of TSP1 (Fig. 1A). We extracted collagens from the dermis of wild-type and *Thbs1*$^{-/-}$ mice with acetic acid, which releases fibrillar collagen molecules polymerized by aldimine cross-links, then measured the hydroxyproline content of these extracts to quantify collagen content. The acetic acid extracts from *Thbs1*$^{-/-}$ samples contained less hydroxyproline, an indication of altered collagen cross-linking, than samples from wild-type mice (Fig. 1B). Analysis by reducing SDS-PAGE and subsequent quantification revealed a significant decrease in collagen α2(I) monomers and altered proportions of cross-linked β1,2 dimers in *Thbs1*$^{-/-}$ samples compared to controls (Fig. 1C-1E). Because both of these features are indicative of altered collagen cross-linking, we examined the status of the major collagen cross-linking enzyme, lysyl oxidase (LOX), by immunoblotting of tissue extracts. The *Thbs1*$^{-/-}$ samples had significantly lower abundance of both proLOX, (the catalytically inactive LOX proprotein), and mature LOX (mLOX), the catalytically active form (Fig. 1F-H). The *Thbs1*$^{-/-}$ samples also tended to show an increased ratio of mLOX to proLOX (Fig. 1I). Collectively, these data implicated both TSP1-collagen I and TSP1-LOX interactions as possible mechanisms underlying the perturbations to collagen fibrils observed in *Thbs1*$^{-/-}$ mice.

TSP1 inhibits proLOX cleavage and binds to multiple fibrillar collagens in vitro

In view of the relative increase in mLOX relative to proLOX in *Thbs1*$^{-/-}$ skin, we examined in vitro whether TSP1 affects the processing of proLOX to mature LOX by the metallopeptidase bone morphogenetic protein-1 (BMP-1) (*5*). In in vitro proLOX cleavage assays, under conditions of partial proLOX cleavage, TSP1 inhibited BMP-1 activity in a concentration-dependent manner but bovine serum albumin (BSA) did not (Fig. 2A, 2B). With regard to the above data and the complex processing of fibrillar procollagen that generates the mature collagen molecule (Fig. 2C), we next tested the binding of native TSP1 isolated from human platelets to different types and forms of fibrillar collagens in vitro by solid-phase binding assays. We tested TSP1 binding to denatured bovine collagen I; pepsin-extracted bovine collagens I and II from skin and trachea, respectively, or pepsin-extracted human collagen III from placenta and native collagen I fibrils isolated from equine tendon. Pepsin cleaves collagen molecules at the inner ends of the N- and C-telopeptides, thus leaving the triple-helical domains in isolation (Fig. 2C). Synthetic triple-helical Gly-Pro-Pro peptide (GPP_{10}) (*45*) and BSA were included as negative controls, and all assays included [Ca^{2+}] and [Zn^{2+}]

in the physiological range. TSP1 bound equally well to pepsin-extracted collagens I, II, or III (Fig. 2D). However, as investigated with regard to collagen I, the binding of TSP1 was related to the molecular form of collagen, because we detected no specific TSP1 binding to denatured collagen I, moderate binding to pepsin-extracted collagen I, and strong binding to native, cross-linked collagen I fibrils that also include the N- and C-telopeptides (Fig. 2D). We also tested binding of TSP1 to recombinant trimers of the collagen C-propeptide domain (C-Pro as shown in Fig. 2C), which drives triple-helix assembly in fibrillar procollagens and also prevents inappropriate fibril formation intracellularly (*2, 4*). We observed concentration-dependent binding of TSP1 to recombinant homotrimers of procollagen α1(I) C-propeptides (CPI), but not to recombinant homotrimers of procollagen α1(III) C-propeptides (CPIII) which shares 66% sequence identity with CPI (Fig. 2E). Binding of TSP1 to CPI depended on the presence of calcium, which is required for homotrimerization of the C-propeptides (*4, 46*) (Fig. 2F). Comparison of the concentration-dependence of TSP1 binding to CPI, pepsin-digested collagen I, or a recombinant, homotrimeric "mini" procollagen I (rProCOL1A1; this protein includes the N-propeptide, N-telopeptide, the 33 most N-terminal and 33 most C-terminal Gly-Xaa-Yaa repeats, C-telopeptide and C-propeptide) demonstrated enhanced binding of TSP1 to the latter protein (Fig. 2G). Thus, TSP1 binding to procollagen I involved the CPI domain and at least one other binding site within the triple-helical region of this fibrillar collagen.

TSP1 binds to specific peptides within the triple-helical domain of collagen II

To identify the putative binding motif(s) for TSP1 within fibrillar collagen triple-helical domains, we screened purified human TSP1 against the Collagen Toolkit II, a library of triple-helical peptides that spans the entire triple-helical domain of homo-trimeric collagen II (*44*), by a solid-phase binding assay similar to enzyme-linked immunosorbent assay (ELISA). Specific Toolkit peptides were identified to bind TSP1 (Fig. 3A). We re-tested these candidate peptides in multiple independent experiments that included the additional negative control of adding the primary and secondary antibodies in the absence of TSP1 (Fig. 3B). Peptides II-5, II-44, and II-52 were confirmed to bind TSP1 in a specific and statistically significant manner (Fig. 3B) compared to GPP_{10}. We also screened Collagen Toolkit III, a library of triple-helical peptides that spans the entire triple-helical domain of homo-trimeric collagen III (*45*) (FIG. S1A), and identified TSP1 binding to peptides III-5, III-52, and III-53 greater than binding to $GPP_{10,}$ but this binding was lower than to the equivalent collagen II peptides (fig. S1B. We tested whether binding of TSP1 to the collagen II peptides was affected by chelation of Ca^{2+} ions, because the 3-D structure of the C-terminal region of TSPs depends on bound Ca^{2+} ions (*47-49*). We found that TSP1 binding to peptide II-44

above background was abolished and binding to II-52 was greatly reduced in the absence of cations. Binding to peptide II-5 was highly variable in the presence of EDTA (Fig. 3C). Similar results were obtained with the Toolkit III peptides (fig. S1C).

In view of a prior report that LOX binds to collagen fibrils (*50*), we also tested for binding of proLOX to TSP1, pepsin-digested collagen molecules, C-propeptides, rProCOL1A1, collagen I fibrils or TSP1-binding collagen triple-helical peptides. Under conditions in which we readily detected proLOX binding to TSP1, no specific binding of proLOX to pepsin-digested collagen molecules or TSP1-binding collagen triple-helical peptides was detected (Fig. 3D). Notably, proLOX bound to CPI and rProCOL1A1, but not to CPIII, indicating the presence of a specific LOX-binding site within the CPI domain of procollagen I (Fig. 3D).

The conserved KGHR site, involved in cross-linking of collagen molecules, is a minimum motif for TSP1 binding

With the exception of peptide II-44, the Collagen Toolkit peptides that bound to TSP1 each contain a common amino acid sequence motif, KGHR (*44*). Therefore, we explored whether TSP1 binding depended on the KGHR motif, first by testing TSP1 binding to shorter derivatives of peptide II-52 that include the KGHR motif. TSP1 bound equally well to peptide II-52a, which contains four guest GXY triplets, peptides II-52b and II-52c, which contain three GXY triplets, and peptide II-52d, which consists of two GXY triplets, thus establishing GLKGHR as a minimum triple-helical region sufficient for TSP1 binding (Fig. 4A). The reduced binding of these short peptides relative to the longer peptide II-52 may reflect conformational differences in these very short regions of native sequence, or a possible positive contribution of a flanking sequence, as observed for the interaction between matrix metalloproteinase 13 and collagen II (*51*). Next, we tested versions of the GLKGHR peptide in which each residue, except for the glycine residues that are required to maintain the triple-helical conformation, were mutated to alanine. Binding to TSP1 was maintained when leucine was replaced with alanine, and was abolished when any one of the lysine, histidine, or arginine residues was replaced with alanine (Fig. 4A). Thus, an intact cluster of positively-charged residues was essential for TSP1-binding. Although not examined further in the context of this study, we noted that peptide II-44 contains the motif RGER and speculate that the closely-spaced arginine residues may explain the binding of TSP1 to this otherwise unrelated peptide.

The KGHR motif occurs at only two sites within fibrillar collagens. The N-terminal and C-terminal KGHR sites are centered at or close to Gly^{88} and Gly^{931}, respectively, of the 1014-residue consensus triple-helical domain. In the fibrillar collagen chains of humans, the N-

terminal motif is fully conserved in all collagens except for COL1A2, COL5A3, COL24A1, and COL27A1, which present variant KGIR, KGQR, KGLK or KGHK motifs, respectively (Fig. 4B). At the C-terminal site, the motif is conserved in most fibrillar collagens, yet is shifted by one GXY triplet and replaced by KGHN in COL1A2 and is disrupted in COL24A1 and COL27A1 (Fig. 4B). KGHR motifs are also substantially conserved in various metazoans (*52*).

KGHR peptides inhibit the binding of TSP1 to native collagen I fibrils

The KGHR motif has a known role in the molecular cross-linking of mature collagen molecules as a target of reactive lysine aldehyde residues in the N- and C-terminal telopeptides that are generated by oxidative deamination by LOX (*6*). To determine if the TSP1-KGHR interaction identified here is physiologically relevant for TSP1 binding to native fibrillar collagen, we tested whether KGHR-containing, triple-helical peptides can inhibit binding of TSP1 to native, cross-linked collagen I fibrils isolated from equine tendons. We found that the non-TSP1–binding peptide II-8 (Fig. 3), had minimal inhibitory activity at all concentrations tested, whereas the KGHR-containing peptide II-52 showed concentration-dependent inhibition with an approximate IC_{50} of 12.5 μM (Fig. 4C). By examining the TSP1 binding by indirect immunofluorescence, we found that, under control conditions, TSP1 bound along the length of the fibrils in a punctate pattern (Fig. 4D, top row). In comparison to fibrils incubated with BSA, the TSP1 antibody reactivity depended on the addition of TSP1 and was sensitive to the concentration of TSP1 (Fig. 4D, top row). Pre-incubation of TSP1 with peptide II-8 before their application to collagen fibrils did not affect binding of TSP1 to fibrils (Fig. 4D), whereas pre-incubation of TSP1 with peptide II-52 substantially reduced TSP1 binding to fibrils (Fig. 4E). We found that an intact KGHR motif was necessary and sufficient for inhibition of TSP1 binding, as established by comparing the activities of GLKGHR, to which TSP1 binds specifically, or GLAGHR, which is not bound by TSP1 (Fig. 4A). Only GLKGHR inhibited binding of TSP1 to fibrils effectively (Fig. 4F). These observations of TSP1 binding were substantiated by quantitative image analysis of fibrils from multiple experiments (Fig. 4G).

TSP1 associates with fibrillar collagens and LOX in human dermal fibroblasts

The above in vitro data and the strong binding of TSP1 to rProCOL1A1 indicated a complex collagen-regulatory mechanism of action for TSP1. Considering that procollagen processing and collagen fibril assembly depend on both intracellular and extracellular events, we turned to human dermal fibroblasts (HDFs) to identify the cellular sites where TSP1, fibrillar collagens, and LOX could potentially interact. Immunoblotting of cell extracts, conditioned

media, and isolated ECM from 24h to 96h cultures of HDFs detected time-dependent abundance of TSP1 in whole cell extracts (fig. S2A), conditioned media and isolated ECM (fig. S2B). Collagen I was detected in cell extracts and conditioned media throughout the time course of the experiments and in ECM at 96h. The CPI fragment was present in media but not in ECM (fig. S2A, S2B). Immunofluorescence using the LOX antibody, which detects both proLOX and mLOX, showed that proLOX predominated over mLOX in cell extracts, whereas mLOX and a sub-fragment thereof were detected transiently in conditioned media. No mLOX was detected in ECM (fig. S2A, S2B). By indirect immunofluorescence and confocal microscopy of HDFs between 48h and 96h after plating, when both intracellular and extracellular TSP1 was present, a major site of colocalization of TSP1 and fibrillar collagens was in intracellular vesicular structures (Fig. 5A, shown for 72h timepoint only). The association changed dynamically with time, increasing between 48h and 72h (Fig. 5B, colocalization per cell quantified by Pearson correlation). Partial, heterogeneous colocalization of extracellular TSP1 with nascent collagen meshworks or protofibrils was also detected in non-permeabilized samples (Fig. 5A and Fig. 5C). At 72h and 96h, cellular LOX (corresponding mostly to proLOX, fig. S2A), colocalized with TSP1 in a subset of intracellular vesicles and colocalized in part with fibrillar collagens in intracellular vesicles (Fig. 5A, 5B). However, extracellular mLOX did not colocalize with collagen fibrils or TSP1 (Fig 5A, 5C).

Because cross-linking of collagen fibrils by mLOX takes place over extended time periods (*53*), HDFs were also examined after 10 days of culture (Fig. 5D). At this time, we observed that the network of fibrillar collagen surrounding the cells was extensive and limited access for antibody staining, as indicated by the limited collagen staining seen with staining of non-permeabilized cells and the detergent-resistant fibrillar network that was revealed by staining after permeabilization (Fig. 5D). In addition to amorphous and punctate deposits of TSP1, extracellular TSP1 colocalized in part with collagen fibrils (Fig. 5D, 5E). We did not detect colocalization of TSP1 and LOX, or LOX and fibrillar collagens, in either permeabilized or non-permeabilized cells (Fig. 5D, 5E). Thus, TSP1-LOX and collagen-LOX associations in HDFs were limited to intracellular sites, where proLOX predominates, and appeared to correlate with early-stage cultures, whereas TSP1 and collagen colocalized in intracellular vesicles and extracellularly over time.

To determine definitively whether fibrillar collagen or LOX associated with TSP1 in HDFs, we carried out in situ proximity ligation assays. This method uses antibodies to detect protein-protein associations in situ within a ~40 nm radius (*54*). In HDFs cultured for 48 to 96 hours, TSP1 clearly associated intracellularly with fibrillar collagens, and this colocalization was limited to vesicular structures (Fig. 5F, 5G; fig. S3A). We found that extracellular association

of TSP1 and fibrillar collagens was restricted to discrete, extracellular patches and fibril-like structures and was maintained over time, reflecting associations within the ECM (Fig. 5F, 5H and fig. S3B). The intracellular association of TSP1 with LOX was also apparent over time (Fig. 5F, 5I, fig. S3A). Extracellularly, in line with the immunofluorescence results, a minor LOX-TSP1 association was detected above background (Fig. 5F, 5J) at times when mLOX was the predominant form of LOX and located extracellularly (fig. S2B). Also in line with the immunofluorescence data, intracellular association of LOX with fibrillar collagen in permeabilized HDFs was minor (fig. S3A), whereas no extracellular association was detected (Fig. 5F, 5K, 5L, fig. S3B). No signal was detected with single antibodies alone, or antibodies to vimentin and TSP1 as a pair of proteins predicted not to associate in HDF (fig S3C). Overall, these experiments in HDFs established that the major sites of colocalization of TSP1 with fibrillar collagen or LOX in HDFs were intracellular, and that TSP1 also associated, over extended periods of time, with extracellular collagen fibrils.

The association of TSP1 and collagen I does not depend on LOX activity

Given the colocalization of TSP1 with LOX in cells and the binding of TSP1 to the KGHR motifs, which are targets of reactive lysine aldehydes in collagen cross-linking, we determined whether inhibiting LOX activity with β-aminoproprionitrile (βAPN) affected the ability of TSP1 to bind to collagen. We found that βAPN treatment altered the ratios of collagen and TSP1 in the deoxycholate-soluble and -insoluble fractions of HDFs and of soluble TSP1 in conditioned medium, whereas cellular LOX abundance was unchanged (Fig. 6A and 6B). As expected, βAPN resulted in fewer extracellular collagen fibrils (Fig. 6C). However, we established by both immunofluorescence (Fig. 6D) and by in situ proximity ligation (Fig. 6E, 6F), that TSP1 remained associated with the residual collagen fibrils. Thus, the interaction of TSP1 with fibrillar collagen did not depend on collagen cross-linking by LOX.

Peptide II-52 inhibits the association between collagen and TSP1 in the ECM of fibroblasts

To investigate the relevance of KGHR-dependent binding of TSP1 to fibrillar collagens in vitro to the associations of TSP1 with collagen in a cellular context, we treated HDFs for 48h or 72h with selected collagen triple-helical peptides at concentrations that effectively competed with collagen I for binding to TSP1 in vitro. By immunofluorescence of non-permeabilized, control HDF cultures, we observed that nascent networks of collagen fibrils started to form after 48h (Fig. 6G), as expected (Fig. 5A). The colocalization of fibrils and TSP1 was retained in HDFs treated with peptide II-2, or the mutants GLAGHR or GLKGAR,

none of which bind to TSP1 (Fig. 6G, 6H). However, we found that collagen fibrils were more fragmentary and aggregated after treatment with either peptide II-52 or GLKGHR, and TSP1-collagen colocalization was decreased (Fig. 6G, 6H). Peptide II-52 was confirmed to decrease the extracellular association of TSP1 and fibrillar collagens by in situ proximity ligation (Fig. 6G, 6I), whereas the short GLKGHR peptide had a minor effect, possibly explained by its lower TSP1-binding activity. Thus, peptides that include an intact KGHR motif compete with cell-derived, collagenous ECM binding to TSP1, and reduced TSP1-binding impacts on the organization of the collagen ECM.

Peptide II-52 Increases myofibroblast differentiation through a TGFβ receptor I–dependent mechanism

Because KGHR-containing peptides perturbed collagen-TSP1 interactions in the ECM of fibroblasts and cell-ECM interactions are important regulators of myofibroblast differentiation (*1*), we examined possible physiological effects of KGHR-containing peptides on fibroblast differentiation. We first established conditions for the induction of two myofibroblast markers: fibronectin containing the alternatively-spliced domain, EDA (EDA-FN), and α-smooth muscle actin (αSMA), by TGF-β1, a known inducer of myofibroblast differentiation in HDFs and measured the abundance of TSP1, LOX, and β-catenin following TGF-β1 treatment (fig. S4A–G). To determine whether TSP1-binding peptides influence the differentiation of myofibroblasts, we measured the abundance of αSMA in cells treated for 96h with the TSP1-binding peptide II-52 or the non-TSP1-binding peptide II-8. II-52 specifically increased the number of αSMA-positive cells, although to a lesser extent than did TGF-β1, whereas peptide II-8 had no effect on the number of αSMA-positive cells (Fig. 7A, 7B). Neither peptide altered HDF proliferation over time relative to vehicle treatment (Fig. 7C). The KGHR motif was important for the induction of αSMA by II-52 because only the wild-type GLKGHR peptide increased the percentage of αSMA-positive cells, whereas the peptides bearing the alanine substitutions in the motif did not (Fig. 7D, 7E). The GLKGHR peptide was less efficient than peptide II-52 at inducing αSMA, likely because of the reduced TSP1-binding activity of GLKGHR compared to II-52. None of the peptides altered HDF cell numbers at 96h (Fig. 7F).

We also investigated whether the signaling process by which II-52 increases the number of αSMA-positive cells is related to that of TGF-β signaling pathways, which are the most widely-studied inducers of myofibroblast differentiation. We treated HDFs with an inhibitor of TGF-β receptor 1 (TGFβR1) kinase activity, SB-431542 (*55*), or an inhibitor of β-catenin signaling, PNU-74654 (*56*), because β-catenin signaling can also promote αSMA

expression. First, we established effective concentrations of these compounds to inhibit the TGFβ1–induced increases in EDA-FN and αSMA protein abundance in HDFs (figs. S4A, S4B). Neither inhibitor affected the abundance of TSP1, but SB-431542 abolished the TGF-β1–induced increase in proLOX and mLOX abundance (figs. S4A, S4C, S4D-G). SB-431542 significantly reduced the induction of αSMA by II-52 as well as by TGF-β1 (Figs. 7G, 7H), whereas PNU-74654 had a small effect on the percentage of αSMA-positive cells only for TGF-β1–treated cells (Figs. 7G, Fig. 7H). Thus, promotion of myofibroblast differentiation by II-52 is mediated by TGF-βR1. To investigate whether this process related to the known role of extracellular soluble TSP1 as an activator of the latent TGF-β1 complex (*28, 40*), we quantified bioactive and total (i.e., following exogenous acid activation) TGF-β1 in media from HDF cultures treated with peptide II-8 or II-52 for 96h. Bioactive TGFβ1 was negligible under all conditions, and the addition of acid to the medium to activate TGF-β1 did not indicate any differences in the abundance of total TGF-β1 between the conditions (Fig. 7I). Thus the activity of II-52 in perturbing extracellular TSP1-collagen interactions also promotes myofibroblastic differentiation by a mechanism that does not relate to altered abundance of extracellular bioactive TGFβ1.

DISCUSSION

The organization and cross-linking of fibrillar collagens is fundamental to tissue integrity. In many tissue settings, TSPs coincide with collagen-rich ECM, which suggests associations that might be highly relevant to normal tissue physiology and fibrotic pathologies. Here we elucidate that TSP1, the prototypic TSP family member, undergoes both intracellular and extracellular molecular interactions with fibrillar collagens. We show the latter to be important for control of myofibroblast phenotype. In vivo, in the dermis of *Thbs1*$^{-/-}$ mice, we observed altered cross-linking of fibrillar collagens, reduced abundance of proLOX and mLOX, and an increased ratio of mLOX to proLOX. We established in vitro that TSP1 bound to proLOX, inhibited the cleavage of proLOX to mLOX by BMP-1, and bound to multiple sites on procollagen I molecules (the C-propeptide domain and the KGHR motifs, that are conserved in the triple-helical domains of collagens II and III). In cultured fibroblasts, TSP1 colocalized with collagen I and, to a lesser extent, LOX, in intracellular vesicles and also associated with extracellular collagen fibrils by binding the KGHR motif. The latter association promoted collagenous ECM organization and likely inhibits myofibroblastic differentiation. Treatment of cells with exogenous, KGHR-containing peptides promoted myofibroblast differentiation through a TGFβR1-dependent process without altering the extracellular abundance of bioactive TGFβ1. These data establish that TSP1 coordinates post-translational collagen I processing and assembly through multiple mechanisms that act at different stages of collagen fibril production, both intracellular and extracellular.

The absence of TSP family members leads to disorganization of collagen fibrils in tissues of mice (*4, 17, 25--27*). By use of binding assays in vitro and demonstrations of protein localisations and colocalisations in cultured cells, we were able to identify specific interactions of TSP1 and then ascribe cellular contexts to these interactions. With regard to proLOX, we identified that TSP1 inhibited BMP-1–mediated cleavage of proLOX in vitro and associated in HDF only with intracellular LOX. Thus, BMP1 activity on proLOX may rise in the TSP1-null context, resulting in altered spatio-temporal aspects of proLOX production and thereby affecting collagen cross-linking. Other studies have reported cardio-protective actions of intracellular TSP4 during endoplasmic reticulum stress within the endoplasmic reticulum of cardiomyocytes, but the status of LOX or collagen ECM organisation was not examined (*57*).

We also investigated whether fibrillar collagens are both binding partners and substrates of LOX. We demonstrated, in vitro, specific binding of proLOX to CPI and rProCOL1A1, but not to CPIII, mature collagens, or KGHR-containing collagen triple-helical peptides. These data

suggest an interaction of proLOX with the CPI region of procollagen I prior to secretion. Consistent with this, collagen and LOX colocalized in HDF; however, in the absence of antibodies specific to proLOX or mLOX we could not determine whether this interaction is specific to proLOX. Free, extracellular CPI is present physiologically and has various functions (*58, 59*), and was detected in early-stage HDF cultures (fig. S2); however, mLOX only appeared in conditioned media after 48h. Our studies of non-permeabilized HDF at 10 days did not detect any collagen-LOX association above background. This is in line with the transient, dynamic nature of LOX enzymatic interactions with collagen telopeptide lysine residues.

With regard to binding of TSP1 to fibrillar collagens, we show that TSP1 exhibits dual direct binding to fibrillar intracellular procollagen I (containing the CPI domain and KGHR sites) as well as to mature, extracellular collagens I, II, and III (via KGHR sites). Given the increased collagen cross-linking in the *Thbs1*$^{-/-}$ context, it is relevant to consider that both mechanisms may limit the rate of homeostatic collagen cross-linking. TSP1 binding to the CPI domain could shield intracellular procollagen molecules from BMP-1 cleavage until secretion, or from active LOX, or both. TSP1 binding to the KGHR motif could protect KGHR motifs from cross-linking by lysine aldehyde attack. We established that TSP1 bound to synthetic KGHR-containing peptides as well as to native cross-linked collagen I fibrils, and that KGHR-containing peptides inhibited TSP1 binding to native fibrils. Hence, the cross-linking of lysine residues does not appear to prevent TSP1 binding at this site. The enhanced TSP1 binding to collagen I fibrils relative to pepsin-digested collagen I suggests that the spatial presentation of KGHR sites may influence extracellular TSP1-binding, perhaps through increased avidity.

Because TSP-1–binding triple-helical collagen peptides disrupted TSP1 binding to native collagen I fibrils (Fig. 4), we were able to explore the functional significance of collagen-TSP1 associations in HDF. We focused on the early timepoints at which we had detected collagen-TSP1 or collagen-LOX associations (as in Fig. 5). KGHR-containing peptides markedly perturbed nascent collagen fibrils, resulting in aggregated collagen “patches”, greater variability in fibril size and shape, and reduced extracellular association of TSP1 and collagen I. KGHR-containing peptides also had specific effects on myofibroblast differentiation (Fig. 7). The induction of αSMA by disruption of the TSP1-collagen interaction (using the TSP1-binding peptide, II-52) required TGFβ-R1 kinase activity, as established using specific inhibitors of either TGF-βR1 or β-catenin signaling. Although extracellular TSP1 is known to activate latent TGFβ1 (*28*), the application of II-52 did not increase the amount of extracellular bioactive TGFβ1 or total active TGFβ1. These results are not

definitive, and the mechanism will require further investigation to determine whether the TSP1-collagen interaction inhibits signaling through TGFβ-R1; for example, by controlling the abundance or activity of TGFβ-R1.

Known molecular partners of fibrillar collagens include fibril-associated collagens with interrupted triple helices (FACITs) (*60*), which align on the surface of the growing fiber and may restrict further recruitment of fibrillar collagen molecule, and the small leucine-rich proteoglycans (SLRPs). Different classes of SLRPs bind to different sites on the surface of collagen fibrils to modulate protofibril nucleation and the subsequent axial and lateral growth of fibrils (*61*). In common with TSP-knockout mice, mice null for individual SLRP family members are viable, but typically show tissue-specific alterations to collagen fibril structures and sizes. For example, mice lacking the SLRP fibromodulin (Fmod) have disorganized and smaller collagen fibrils in tendons and increased C-telopeptide cross-linking of collagen I (*62-64*). Fmod is proposed to facilitate extracellular collagen cross-linking because it binds to KGHR motifs in fibrillar collagens and binds and activates LOX without affecting LOX maturation (*65, 66*). TSP1 and Fmod are the only ECM proteins identified to date that bind to the KGHR motif of fibrillar collagens, though binding of PEDF (pigment epithelium-derived factor) to collagen I depends on the arginine residue in this motif (*67*). Thus, TSP1 and Fmod bind the triple-helix of fibrillar collagens at a site distinct from the motifs recognized by the major collagen receptors and other collagen-binding proteins (6*8-70*).

Overall, the collective action of many ECM proteins on fibrillar collagens emphasizes the evolution of a complex network of mechanisms to control collagen fibril assembly and the activity of LOX. The central conceptual advance of this study is the identification of an additional form of molecular control of fibrillar collagens. Our data reveal actions of TSP1 on fibrillar collagen, exemplified here by collagen I, at several steps during the molecular processing and assembly of collagen fibrils. Detailed studies will be needed to determine whether these activities influence procollagen trafficking, mask KGHR cross-linking sites, or control the spatio-temporal positioning of proLOX or its access to collagen molecules prior to secretion. The mechanism of action of TSP1 is unique in regulation of collagen homeostasis because it acts on both intracellular procollagen and proLOX as well as on extracellular fibrillar collagen.

MATERIALS AND METHODS

Materials and Cells

Normal human dermal fibroblasts (HDFs) from foreskins of healthy juveniles (C-12300, Promocell) were cultured in fibroblast growth medium (C-23010, Promocell) with 50μg/ml L-ascorbic acid and used for experiments between passages 3 and 8. Primary antibodies were used for detection of α-smooth muscle actin (SMA), collagen I, EDA-fibronectin, TSP1; vimentin, collagen I, collagen α1 C-propeptide (CPI) (LF41) (*71*), and lysyl oxidase (LOX). Full details for each antibody are given in Table S1. Secondary antibodies were as follows: HRP-conjugated antibody recognizing mouse IgG or rabbit IgG; FITC-conjugated antibody for mouse IgG; TRITC-conjugated antibody for rabbit IgG (Table S2). Chemicals used and supplier information are listed in Table S3. The following inhibitors were used: βAPN, SB431542, and PNU-74654 (full details are in Table S4). Purified and recombinant proteins used are listed in Table S5. Buffer compositions are given in Table S6.

Sample preparation and collagen extraction from mouse skin

Collagen was extracted from the skin of 8 week-old wild-type C57BL/6 male mice (JAX 000664, Jackson Laboratory), n=4, and B6.129S2-*Thbs1*^{tm1Hyn}/J male mice (JAX 00614, Jackson Laboratory), n=4. JAX 00614 mice were backcrossed to C57BL/6J for 8 generations prior to establishment of the stock at Jackson Laboratory (*19*). 16 cm^2 of skin was dissected from each mouse, shaved, any underlying adipose removed and samples cut into 3mm-wide strips. 2g skin/mouse was incubated with PBS containing protease inhibitor cocktail (2 mM NEM, 10 mM leupeptin, 20 mM pepstatin A), under rotation at 4ºC overnight and extracts collected as supernatants after centrifugation at 14000xg for 30min at 4ºC. Pellets were resuspended in 3ml of 0.5M acetic acid (HAc) containing protease inhibitor cocktail and incubated under rotation for 24h at 4ºC. Supernatants were collected after centrifugation at 14000xg for 30min at 4ºC to obtain the HAc-soluble fraction and Trizma base was added to bring the pH to 7, followed by hydroxyproline quantitation. Aliquots of acid-soluble collagens from each mouse, (corresponding to 250μmol of hydroxyproline), were boiled in reducing SDS-PAGE sample buffer prior to analysis by SDS-PAGE.

Hydroxyproline Quantitation

Acid-extracted collagens were brought to a concentration of 600μg/ml and subjected to hydrolysis with 6M HCl for 20h at 95ºC. Samples were centrifuged at 14000xg for 10min and supernatants diluted in dH_2O to a final concentration of 4M HCl. Hydroxyproline assay kit was used according to manufacturer's procedures (QuickZyme, Biosciences).

ECM Proteins and Collagen Peptides

The following proteins were used (details, catalogue numbers and suppliers of proteins are listed in the Supplementary Table S5): recombinant human TGFβ1; purified human thrombospondin-1 (TSP1); recombinant human lysyl oxidase (proLOX); recombinant human mini-pro-collagen I alpha 1 (rProCOL1A1); denatured bovine collagen I; pepsin-digested collagens I, II, III; native collagen I fibrils; recombinant human procollagen C-propeptide I (CPI) and III (CPIII) homotrimers (*4, 72*). Collagens were dissolved overnight in 0.5M HAc to final concentrations of 1mg/ml or 5mg/ml. Collagen Toolkit II and derivative peptides were synthesised as C-terminal amides on TentaGel R RAM resin using Fmoc chemistry (*44*), but using CEM Liberty or Liberty Blue microwave-assisted solid phase synthesisers. Each guest sequence (27-residues for Toolkits or shorter sequences containing KGHR and its derivatives as indicated) is placed between GPC$(GPP)_5$- and -$(GPP)_5$GPC host sequences to stabilise the triple-helical conformation. GPP_{10}, a negative control peptide (GPC-$[GPP]_{10}$GPC-NH_2), represents the combined host sequence. All peptides were dissolved in 10mM HAc at 5mg/ml final concentration.

Solid-Phase Binding Assays

Collagen triple-helical peptides at 10μg/ml in 10mM acetic acid were coated onto wells of 96-well plates (Immulon 2HB, 3455, ThermoFisher Scientific) overnight at 4°C. Collagen fibrils, pepsin-digested collagens, or denatured collagen I were adsorbed at saturating concentration of 10μg/ml in 10mM acetic acid. In other experiments, wells were coated overnight at 4°C with TSP1 diluted in TBS containing 2mM $CaCl_2$, or CPI or CPIII diluted in TBS/0.5mM $CaCl_2$, each to a final concentration of 24nM or as indicated in individual figure panels. rProCOL1A1 was diluted in 10mM acetic acid to a final concentration of 24nM. All following steps were at room temperature (RT). Wells were blocked with 50μg/ml BSA Fraction V. Test proteins in solution and primary or secondary antibodies were each diluted in Incubation Buffer (IB; Supplementary Table S6), containing either 2mM EDTA, or 2mM $CaCl_2$ and 15mM $ZnSO_4$. Antibodies were diluted as given in Supplementary Tables S1 and S2. All steps were performed for 1h, and each followed by three washes in IB. Final concentrations of proteins in solution were 8nM TSP1, or 24nM of rProCOL1A1, CPI or CPIII. For colorimetric detection, 100 μl of a 1:1 mixture of TMB and 0.02 % H_2O_2 in citric acid buffer was added. Reactions were stopped with 100 μl of 2M H_2SO_4 and absorbance measured at 450nm in a 96-well plate reader (M2/spectra max; Molecular Devices). As a negative control to confirm the specificity of each primary antibody, 8nM BSA was added in IB instead of the test protein. For competition assays, TSP1 was pre-incubated with increasing amounts of Toolkit peptides II-8, II-52 or selected derivatives (II-52d, II-52dK3A) for 1 h prior to adding to wells coated with collagen I fibrils and assays developed as described above.

ProLOX cleavage

Recombinant proLOX (50 nM) was incubated without or with 0.18 nM recombinant human BMP-1, either in the presence of either 225nM BSA, or TSP1 at concentrations of 25nM, 50nM, 110nM or 225nM, at 37 ºC for 15 minutes. Reactions were ended by addition of an equal volume of SDS-PAGE sample buffer and samples resolved on 10 % polyacrylamide gels under reducing conditions and immunoblotted for LOX or TSP1. ProLOX cleavage was quantified by measuring the pixel intensities of proLOX and mLOX bands using ImageJ software. Percentage of LOX cleavage was then calculated as follows: % of LOX cleavage = [(mLOX/proLOX)lane / (mLOX/proLOX) from BMP1+ BSA condition] X 100.

Fluorescence Microscopy

For binding of TSP1 to fibrillar collagen, glass coverslips were coated with 60μl of native collagen I fibrils (2μg/ml in 0.01M HAc) overnight at 4°C. In some experiments, TSP1 (8nM in IB), was incubated with selected collagen triple-helical peptides for 1h at RT. Peptides used were: II-8, II-52, II-52d (GLKGHR peptide), or II-52dK3A (GLAGHR peptide), each at 45μM in 0.01M HAc, or the equivalent volume of 0.01M HAc as a negative control. All ligands and antibodies were diluted in IB containing 2mM $CaCl_2$ and 15μM $ZnSO_4$. In parallel, collagen-coated coverslips were blocked with 5% BSA and then TSP1, without or with peptide, added for 1h, followed by anti-TSP1 for 1h, and then FITC-conjugated antibody to mouse IgG for 1h. All steps were followed by three washes in IB. Coverslips were washed and mounted in Vectashield (Vector Laboratories), and examined under a Leica DMI6000 inverted epifluorescence microscope with a HCX PL APO 100X 1.40NA oil objective. XY images were captured with a DFC365FX Leica monochrome CCD camera run by Leica Application Suite X software (v3.0.2). TSP1 bound to collagen fibrils was quantified with Volocity 6.3 Software by: 1. selecting areas in phase contrast images that corresponded to collagen fibrils by finding objects with intensity values between 0 (lower) and 60 (upper); 2. finding objects bigger than 4μm^2 under the green fluorescence channel within the above fibril areas. The total fluorescence intensity in each area was then normalized over the pixel counts for the collagen fibril area.

For localization of collagen I, lysyl oxidase, αSMA and TSP1 in HDF, ~~HDF (~~3x10^4 cells per well were plated onto 13mm glass coverslips for 48, 72 or 96h at 37°C. In some experiments, cells were treated with 15 μM collagen Toolkit II peptides for either 48, 72 or 96h. In other experiments, HDF were treated with 10 μM SB-431542, 10 μM PNU-74654 or the equivalent volume of DMSO for 30mins prior to addition of collagen Toolkit II peptides or 2ng/ml TGFβ1 for 96h, or treated with 2 mM βAPN for 10 days. For co-localization in non-permeabilized cells, coverslips were fixed in 4% PFA. For permeabilization, cells were either

treated with methanol/acetone (1:1) or fixed in 2% PFA and permeabilized in 0.5% Triton-X100 in PBS. All following steps were carried out at RT in a humidified chamber and each step was followed by three washes in PBS. Coverslips were blocked with 1% BSA for 30 min, then cells stained with the appropriate primary antibody(s) diluted in 2% BSA for 1h 30min, and incubated for 1h with FITC-conjugated antibody to mouse IgG and then with TRITC-conjugated antibody to rabbit IgG, each diluted in PBS containing 5% BSA. Coverslips were washed and mounted as above. Cells were examined under an inverted Leica SP5-AOBS confocal laser-scanning microscope, with a HCX PL APO lambda blue 63x 1.4NA oil objective. XY images were captured as Z stacks with a 0.25 μm Z step size, with photomultiplier tube (PMT) detectors with a photocathode made of Gallium-Arsenide-Phosphide (GaAsP) (Leica) for collecting light emission. Images were captured with Leica Application Suite AF software (v2.7.3.9723). For colocalization studies, Pearson correlation was measured in Volocity 6.3 software by the method of Costes to set automatic thresholds (*73*). At least 36 cells were analyzed per condition, per experiment. For non-permeabilized cells, Pearson correlation was measured per field; a minimum of 6 fields was taken for each condition in each independent experiment. For permeabilized cells (48h – 96h cultures), the Pearson correlation was measured for each cell. Around 80 cells were analyzed for each condition in each independent experiment. In 10 days cultures, nuclei could not be counted accurately due to dense ECM and over-lapping of cells, therefore the Pearson correlation is reported per field.

For in situ proximity ligation, HDF were plated onto 13mm glass coverslips, fixed, and incubated with primary antibodies as described above. In situ proximity ligation assays were performed using DUOlink (Sigma) according to the manufacturer's instructions. Briefly, secondary antibodies coupled to cDNA probes were added for 1h at 37°C, then fluorescent detection was performed with Detection Reagents Green. Coverslips were examined under a Leica wide-field microscope (Leica DMI4000 B) and a HCX PL APO 63X 1.40NA oil objective. XY images were captured with a DFC310 FX Leica digital camera run by Leica Application Suite (LAS) X software (v4.5). Images were analyzed in ImageJ (Fiji). The intensity range was set between 37-255 intensity values. The total area of particles/field was measured and, dependent on the experimental design, the number of nuclei per field counted.

Preparation of Cell Lysates and Supernatants

HDF ($4x10^5$ cells) were cultured for 24, 48, 72 or 96 hours at 37°C. In some experiments cells were treated with 10 μM SB-431542 or PNU-74654 or DMSO for 30min prior to addition of 2ng/ml TGFβ1 for 96h. Cells were lyzed in SDS-PAGE sample buffer to obtain total cell

extract, or with 2% deoxycholate buffer. Conditioned medium (CM) was harvested and mixed 1:1 with SDS-PAGE sample buffer. In parallel, heparin-binding proteins were collected from CM by incubation with 25μl of Affi-gel® heparin beads for 90min with rotation at 4°C, followed by washing in PBS and resuspension of beads in SDS-PAGE-SB. For measurements of TGFβ1 activity, HDF were plated as for immunofluorescence microscopy and media harvested after 96h of culture, centrifuged to remove cell debris and 100μl aliquots analyzed for active TGFβ1 by TGFβ1 Enzyme Immunoassay (Enzo, ADI-900-155), without or with acid activation of TGFβ1, according to manufacturer's procedures.

SDS-PAGE and Immunoblotting

Proteins were resolved on 10% or 7.5% polyacrylamide SDS-polyacrylamide gels under reducing conditions. Gels were stained with Gel Code Blue Stain reagent. For immunoblotting, proteins were transferred to PVDF membrane (ISEQ00010, Immobilon-P, Millipore) and blocked in immunoblot Blocking Buffer (BB) overnight at 4°C. Membranes were probed with primary antibodies diluted in BB for 90min at RT under agitation. After three washes in BB, membranes were probed with appropriate HRP-conjugated secondary antibodies for 1h at RT, washed and incubated with Amersham ECL and signals detected on X-ray film (28906836, GE Healthcare). Protein levels from Coomassie-stained gels and immunoblots were quantified with NIH ImageJ software (Fiji), by boxing each lane and generating histogram plots showing peak areas corresponding to each band along the lane.

Multiple sequence alignment

Collagen sequences were obtained from NCBI proteins division. Multiple sequence alignments were prepared in MUSCLE 3.8 (*74*) at default parameters through the resources of EMBL/EBI (http://www.ebi.ac.uk/Tools/msa) and are presented in BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Statistical analysis

All experiments were carried out at least 3 times independently unless stated otherwise in the figure legend. Statistical tests were made in GraphPad Prism v5.01. Data were analyzed with D'Agostino-Pearson omnibus and Shapiro-Wilk tests to check for normal distribution. More than two groups of data were analyzed by one-way ANOVA with Bonferroni's multiple comparison test. Where distribution was not normal, two groups of data were analyzed by non-parametric Mann-Whitney test. More than two groups of data were analyzed by non-parametric Kruskal-Wallis test with Holm's correction. In the figures, *** = p-value ≤ 0.0005; ** = p-value ≤ 0.005; * = p-value ≤ 0.05.

Ethical Standards
The experiments comply with the current laws of the country in which they were performed.

SUPPLEMENTARY MATERIALS

Fig. S1. Identification of specific TSP1-binding peptides within the triple-helical region of collagen III.

Fig. S2. Detection of collagen I, TSP1, fibronectin, LOX and collagen I C-propeptide in cell fractions, conditioned media, and ECM isolated from human dermal fibroblasts.

Fig. S3. Detection of the association of TSP1 with collagen I, collagen II, and LOX in HDF by in situ proximity ligation.

Fig. S4. TGF-β1–mediated induction of αSMA in HDF.

Table S1. Primary antibodies and the dilutions used in this study.

Table S2. Secondary antibodies and the dilutions used in this study.

Table S3. Chemicals used in this study.

Table S4. Chemical inhibitors used in this study.

Table S5. Proteins used in this study.

Table S6. Buffers used in this study.

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FIGURE LEGENDS

Figure 1. Fibrillar collagens and LOX in skin samples from wild-type and *Thbs1*$^{-/-}$ mice. (**A**) Immunoblots showing TSP1 in spleen samples from wild-type (WT) and *Thbs1*$^{-/-}$ mice (4 mice of each genotype, numbered 1-4). Ponceau staining of the gel used for immunoblotting is shown as a loading control. (**B**) Hydroxyproline content of acetic acid extracts from WT and *Thbs1*$^{-/-}$ skin samples. N = 4 for each genotype. (**C**) SDS-PAGE analysis of acid-extracted collagens from skin of WT and *Thbs1*$^{-/-}$ mice under reducing conditions. The samples were equalized for hydroxyproline content. Bands corresponding to various forms of collagen trimers, dimers, and monomers are noted. N = 4 for each genotype. (**D**) Quantification of collagen α2(I) in WT and *Thbs1*$^{-/-}$ samples. N = 4 for each genotype. (**E**) Proportions of different collagen forms extracted from skin of WT and *Thbs1*$^{-/-}$ mice, including mean ± S.D. N = 4 animals per genotype. (**F**) Immunoblot showing proLOX and mLOX in total skin extracts from WT and *Thbs1*$^{-/-}$ mice. Ponceau staining of the gel used for immunoblotting is shown as a loading control. N = 4 for each genotype. (**G**, **H**) Quantification of proLOX (G) and mLOX (H) from immunoblots, normalized to the major loading control band. N = 4 for each genotype. (**I**) mLOX/proLOX ratio from the quantitations in panels G and H ($p = 0.057$). In each scatter plot, the bar indicates the mean. Data were analyzed by Mann-Whitney U-test. Each dot in panels B and D represents the value from an individual animal. Each dot in panels G, H, and I represents the mean of 4 separate measurements from each animal.

Figure 2. TSP1 binds to proLOX and fibrillar collagens I, II and III in vitro.
(**A**) Representative immunoblots of assays for cleavage of recombinant human proLOX by BMP1 in the absence or presence of increasing concentrations of TSP-1, as indicated. BSA was used as a negative control. N = 3 independent experiments. (**B**) Quantification of the concentration-dependent inhibition of BMP-1–mediated proLOX cleavage by TSP1. Each data point represents the mean from 3 independent experiments, and the error bars indicate s.e.m. (**C**) Schematic diagram of the domains of a fibrillar procollagen molecule. (**D**) Binding of TSP1 to immobilized collagens in solid-phase binding assays. Col I (denatured), Col I, Col II, and Col III samples were derived from pepsin-digested collagen preparations. GPP_{10} and BSA were included as negative controls. One-way ANOVA and Bonferroni's multiple comparison test was performed against GPP_{10}. N = 4 independent experiments**.** (**E**) Concentration dependence of TSP1 binding to the indicated immobilized proteins (ligands). The ligands (CPI or CPIII) were immobilized and incubated with or without TSP1 (T). A mouse antibody recognizing TSP1 (C9) and goat anti-mouse antibody (GAM) were included

in each reaction to quantify binding. N = 4 independent experiments**.** (**F**) Solid-phase binding assays testing TSP1 (T) binding to immobilized CPI or BSA in the presence of either Ca^{2+} or EDTA and quantified with the antibodies C9 and GAM. N = 4 independent experiments**.** One-way ANOVA and Bonferroni's multiple comparison test was performed against BSA. (**G**) Concentration dependence of TSP1 (T) binding to the indicated immobilized proteins (ligands), rhProCOL1A, Col I, and CPI. Antibodies C9 and GAM were used to quantify binding. N = 4 independent experiments**.** Dotted lines in E and G indicate negative control assays without TSP1. Data points in D–G indicate the mean, and error bars indicate the s.e.m.

Figure 3. Identification of specific TSP1-binding peptides within the triple-helical region of collagen II. (**A**) Quantification of the binding to TSP1 to the 56 peptides (numbered for every fifth peptide as II1, etc) of the Collagen Toolkit II in the presence of Ca^{2+} and Zn^{2+}. The horizontal line represents the background binding to GPP_{10}. Each bar represents the mean of duplicate samples. (**B**) Binding of TSP1 (T) to specific TSP1-binding peptides as identified from the initial hits in Toolkit II in the presence of Ca^{2+} and Zn^{2+}. Binding was quantified by indirect colorimetric assay using the antibodies C9 and GAM. N = 4 independent experiments. (**C**) Quantification of the binding of TSP1 (T) to the indicated peptides in the presence of the Ca^{2+} chelator EDTA. N = 4 independent experiments**.** (**D**) Binding of proLOX to immobilized TSP1 and the indicated proteins and peptides derived from fibrillar collagen. N = 4 independent experiments. In all panels, GPP_{10} and BSA were included as negative controls. Pepsin-digested collagens I and III were used as positive controls in B and C. In B–D, one-way ANOVA and Bonferroni's multiple comparison tests were performed against $GPP_{10.}$ Each bar indicates the mean, and error bars indicate the s.e.m.

Figure 4. KGHR is a minimal and conserved motif for TSP1 binding.
(**A**) Binding of TSP1 (T) to derivatives of TSP1-binding peptides from the Collagen Toolkit II, truncated or mutated as indicated (guest peptides). Binding was quantified by indirect colorimetric assay using the antibodies C9 and GAM. Data are from 5 independent experiments. (**B**) Alignment showing the KGHR motifs (red text) in the N-terminal and C-terminal triple-helical regions of the indicated collagen chains of *Homo sapiens* (Hs). The numbers 87 and 930 refer to the amino acid position in the triple-helical domain of collagen α1(I). Black shading, identical residues; grey shading, conservative substitutions; no shading, no conservation. (**C**) Quantification of TSP1 binding to the indicated Collagen Toolkit II peptides II-8 or II-52*.* Each data point represents the mean, and bars represent the s.e.m. of 3 independent experiments. (**D–F**) Merged phase-contrast and

immunofluorescence images of TSP1 binding to collagen I fibrils isolated from tendons (D); TSP1 binding to collagen I fibrils in the presence of the indicated Collagen Toolkit II peptides (E), or in the presence of peptides of the indicated sequence (F). Images are representative of 4 independent experiments. Scale bar, 15 μm. (**G**) Quantification of immunofluorescence staining for TSP1 bound to collagen fibrils, calculated as total fluorescence intensity within fibrils normalized on pixel count within fibrils. Each dot represents the value from an individual experiment, each bar indicates the mean, and the error bars indicate the s.e.m. from 4 independent experiments. Data were analyzed by Kruskal-Wallis test and pairwise tests against BSA with Holm's correction.

Figure 5. Localization of TSP1, fibrillar collagen and LOX in human dermal fibroblast cultures. (**A**) Dual indirect immunofluorescence staining of the indicated pairs of proteins in permeabilized or non-permeabilized human dermal fibroblasts (HDF) cultured for 72h. The regions shown at higher magnification in the inset panels are boxed by dotted lines in the main panels. Nuclei were stained with DAPI (blue). Images are representative of 4 independent experiments. (**B**) Quantification by Pearson correlation (per cell) of colocalization of the indicated pairs of proteins in permeabilized (P) HDF cultures at the indicated times. C, collagen I; T, TSP1; L, LOX. N = 4 independent experiments with at least 36 cells analyzed per condition per experiment. Data were analysed by Kruskal-Wallis test and Dunn's comparison. (**C**) Quantification by Pearson correlation (per field) of colocalization of the indicated pairs of proteins in non-permeabilized (NP) HDF cultures at the indicated times. N = 4 independent experiments with at least 6 fields analyzed per condition per experiment. (**D**) Dual indirect immunofluorescence staining of the indicated pairs of proteins in permeabilized and non-permeabilized HDF after 10 days of culture. Images are representative of 4 independent experiments. The regions shown at higher magnification in the insets panels are boxed by dotted lines in the main panels. (**E**) Quantification by Pearson correlation (per field) of colocalization of the indicated pairs of proteins in HDF after 10 days of culture. N = 4 independent experiments with at least 6 fields analyzed per condition per experiment. (**F**) In situ proximity ligation assay for the indicated pairs of proteins in HDF cultured for 72h. Images are representative of 4 independent experiments. (**G-L**) Quantification of in situ proximity ligation signals for the indicated pairs of proteins over time in permeabilized (P) or non-permeabilized (NP) HDF. 2Ab, secondary antibodies only. Each data point represents the mean from one experiment, and the bars indicate the mean. N = 4 independent experiments (G–J), N = 3 independent experiments (K–L). At least 36 cells were analyzed per condition for each experiment. Panels A, D and F show results for HDF at passage 7 and are representative of 4 experiments on HDF between passages 4 and 8. Scale bars, 50 μm (main image) and 10 μm (insets)**.**

Figure 6. Effects of LOX inhibition or KGHR-containing peptides on the localization of TSP1 and fibrillar collagen in human dermal fibroblast cultures. (**A**) HDF were cultured for 10 days in the absence or presence of the LOX inhibitor βAPN. The soluble and insoluble fractions of cell extracts and conditioned medium (CM) from each treatment group were analysed by immunoblotting for collagen I α(I), TSP1, and proLOX. GAPDH is a loading control. Molecular markers are indicated to the left of the blots in kDa. (**B**) Quantification of the indicated proteins from the immunoblots in (A). Protein abundances were normalized to GAPDH and expressed as a ratio versus control cells. Each bar represents the mean of 3 independent experiments, and the error bars indicate s.e.m. Mann-Whitney tests were performed for each pair (not significant). (**C**) Indirect immunofluorescence images showing TSP1 and collagen I in HDF cultured for 10 days in the absence or presence of βAPN. Nuclei were stained with DAPI (blue). Images are representative of 4 independent experiments. The regions shown at higher magnification in the insets panels are boxed by dotted lines in the main panels. (**D**) Quantitation by Pearson correlation (per field) of colocalization of TSP1 and collagen I under the indicated conditions. N = 4 independent experiments with at least 36 cells analyzed per condition per experiment. (**E**) In situ proximity ligation assays in permeabilized HDF after 10 days culture in the absence or presence of βAPN. Images are representative of 4 independent experiments. (**F**) Quantitation of proximity ligation signals under the indicated conditions. N = 4 independent experiments with at least 36 cells analyzed per condition per experiment. (**G**) Immunofluorescence showing collagen and TSP1 in HDF cultured and treated for 48h with the indicated peptides, then fixed and stained by indirect immunofluorescence (IF) or in situ proximity ligation (Prox). HAc, acetic acid. In the upper row, the regions shown at higher magnification in the insets panels are boxed by dotted lines in the main panels. Each row is representative of 4 independent experiments. (**H)** Quantification by Pearson correlation of immunofluorescence colocalizations as in G. N = 4 independent experiments with at least 36 cells analyzed per condition per experiment. (**I**) Quantification of proximity ligation signals for conditions as in G. N = 4 independent experiments with at least 36 cells analyzed per condition per experiment. Data in H and I were analyzed by Kruskal-Wallis test and pairwise tests against HAc with Holm's correction. Scale bars, 50 μm (main image) and 10 μm (insets)**.**

Figure 7. KGHR-containing, TSP1-binding collagen triple-helical peptides increase myofibroblast differentiation through a TGF-βRI–dependent mechanism.

(**A**) Merged immunofluorescence images of HDF cultured for 96h in the presence of HAc, TGF-β1, collagen peptide II-8, or collagen peptide II-52 and stained for αSMA (green) and nuclei (DAPI, blue). Images are representative of 3 independent experiments. (**B**) Quantification of the percentage of αSMA-positive cells under the indicated conditions from 3 independent experiments. (**C**) Effects of the various treatments on cell numbers over time, N = 3 independent experiments. (**D**) Merged immunofluorescence images showing αSMA (green) and DNA (blue) in HDF cultured for 96h in the presence of HAc, TGF-β1, or the indicated peptides. Images are representative of 3 independent experiments. (**E**) Quantification of αSMA-positive cells per field following treatment with the indicated peptides. N = 3 independent experiments. (**F**) Effects of the indicated peptides on cell numbers at 96h. N = 3 independent experiments. (**G**) Merged immunofluorescence images showing αSMA (green) and DNA (blue) in HDF cultured for 96h in the presence of the inhibitor of TGFβR1 kinase activity SB-431542 (SB) or the β-catenin signaling inhibitor PNU-74654 (PNU). Images are representative of 3 independent experiments. (**H**) Quantification of αSMA-positive cells after 96h in the presence of the indicated inhibitors. N = 3 independent experiments. (**I**) Quantification of bio-active and total (determined after acid activation) active TGFβ1 in media from HDF cultured in the presence of the TSP1-binding peptide II-52. N= 3 independent experiments with duplicate samples per condition per experiment. In all graphs, each data point indicates the mean, and error bars indicate the s.e.m. In B, C, E, F and H, at least 30 cells were scored per condition per experiment. Data were analyzed by Dunnett's multiple comparison test (one-way ANOVA) against HAc (B) or by Kruskal-Wallis test and pairwise tests against HAc (E and F), or against DMSO (H). Scale bars, 50 μm (main image) and 10 μm (insets)**.**